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The Chronic Effects of Estradiol Benzoate on Ventricular Myocardial Blood Flow in the Adult Female Rat

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This is to certify that the thesis prepared by Stephen Maurice Ashe entitled "The Chronic Effects of Estradiol Benzoate on Myocardial Blood Flow in the Adult Female Rat" has been approved by his committee as satisfactory completion of the thesis requirement for the degree of Master of Science in Biology.

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I wish to dedicate this thesis to my parents who deserve only the best things in life and are very special to me.

Abstract

The Chronic Effects of Estradiol Benzoate on Ventricular Myocardial Blood Flow in the Adult Female Rat.

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Clinical and experimental data suggest that humans and some animals are protected from acute myocardial infarction by estrogen. There has been much speculation of the exact role which estrogen plays in the circulation. The mechanism of this hormonally related protection is unclear.

In the present study the chronic effects of estradiol benzoate on coronary blood flow in adult female rats were studied. Female control rats, castrates and castrates receiving estrogen for a period of 16 weeks were used. In vivo tracer microsphere studies of myocardial blood flow allowed the quantification of the effects of estrogen on coronary circulation. Results of this work demonstrated that levels of estrogen significantly influence blood flow in ventricular myocardium.

Literature Review

Perhaps the most general effect of estrogens (Figure 1) is to proliferate tissue growth by stimulating cell division (Turner and Bagnara, 1976). Much attention, however, has been focused on estrogen and its relationship to coronary atherosclerosis (Blumgart, 1968). Clinical and experimental evidence suggest that physiological levels of estrogens mitigate or postpone many circulatory ailments such as hypertension, coronary artery disease, myocardial ischemia and certain pathologic changes in the electrocardiogram (Stumpf et al., 1977).

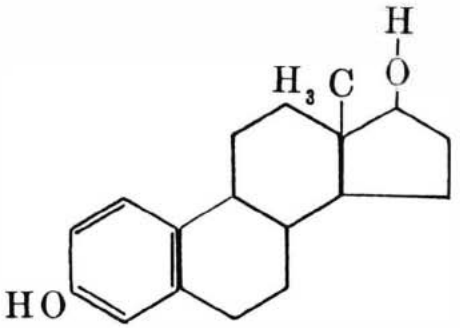
There is evidence that cardiovascular disease is more common in men than women in the United States. This data has been ascribed to both stress in males and the effects of protective hormones in females (Stumpf et al., 1977). Treatment with estrogen has been observed to strongly exert a beneficial effect of significantly prolonging survival and decreasing the mortality rate by 50% in men who have already experienced an acute myocardial infarction (Stamler et al., 1963). How estrogen exerts this effect is not known. Conversely, however, McCall et al. (1980) has contended that exogenous estrogen increased mortality in men with a pervious myocardial infarction.

Rona et al. (1963) and Wexler (1969) have shown that the male rat is more susceptible than the female to the necrogenic actions of isoproterenol. Medically, isoproterenal is a β-adrenergic stimulant

Figure 1. Representation of the molecular structure of Estradiol 17β.

ESTRADIOL 17 β

$C_{18} H_{24} O_2$



that in small doses is a positive inotrope and a positive chronotrope; in large doses this drug produces cardiac toxicity (Melmon and Morrelli, 1972). It was suggested that the increased susceptibility of the male rat may result from the fact that the male rat weighs more than the female at any given age (Rona et al., 1963). McGrath and Martin (1975) found that the female Japanese Quail, which consistently weighs more than the male, has a much greater resistance to myocardial necrotic agents. This finding disputes the weight-basis hypothesis. It also was demonstrated that the exogenous administration of estrogen can protect the male Japanese Quail from necrotic lesions of the heart induced by isoproterenol. The mechanism by which estrogen may exert its protective effect in this instance is unclear.

Higano et al. (1963)noted that estrogen had induced augmented resistance to cardiovascular disease after two-year estrogen replacement therapy in castrated human females. Oophorectomized women without this replacement therapy displayed accelerated artherosclerosis in the form of myocardial infarctions, peripheral vascular disease and cerebral thrombosis.

The mechanisms by which estrogens exert their protective effects are not known and estrogens are new factors in coronary artery disease whose roles need to be defined (Lancet, 1977). It has been demonstrated that a decrease in uterine, renal and peripheral vascular resistance accompanies pregnancy and that this decrease in resistance can be duplicated by the experimental administration of estrogen (Sullivan, 1974). Estrogen acting on smooth muscle of the blood vessels causes vasodilation in the uterine area. Estrogen also may play an important role as a relaxer of the umbilical cord artery favoring its relaxation (Silva De Sa' and Meirelles, 1977). Jaffe (1977) however has postulated

that estrogen induces an increase in smooth muscle tone of the coronary artery. Brown et al. (1974) has noted that peaks in uterine blood flow during the estrous cycle of the ewe coincide with cyclic surges of estrogen secretion, and that these blood flow changes can be reproduced experimentally by estrogen and progesterone administration. Thus estrogen has been shown to be a potent vasodialator in various vascular beds (Sullivan, 1974) and (Brown et al., 1974), but its specific action on myocardial blood flow has not been evaluated or described.

Autoradiographic studies of the rat heart have revealed that estradiol concentrates in the cell nuclei of the myocardium of the atria and auricles, as well as the myometrium of the uterus. It has also been demonstrated, with studies utilizing (^{3}H) estradiol, that estradiol has target sites in the walls of blood vessels in heart muscles (Stumpf et al., 1977).

Although the physiological basis is not known, premenopausal women have a lower incidence of death from myocardial infarction than men (Stamler, 1967), but many investigators postulate that estrogen itself is unlikely to provide this protection. This assumption is supported by the findings that estrogen administration at contraceptive levels has been associated with an increase in incidence of both myocardial infarction (Robinson et al., 1963) and stroke (McDowell et al., 1967). The same associations have also been found for estrogen used in conjunction with progestogens at oral contraceptive levels for myocardial infarction (Waxler et al., 1971) and stroke (Horenstein, 1975).

The results of some of these studies are strongly supportive of estrogen effecting the cardiovascular system. Though some of the studies support that estrogen does effect the incidence of myocardial infarction, most of the studies fail to explain why these results occur.

Many of the studies used contraceptive levels of estrogen (Jaffe, 1977; Robinson et al., 1963; Waxler et al., 1971). The studies did not evaluate how estrogen effects blood flow to the myocardium. There is a need for studies to be conducted using normal physiologic replacement levels of estrogen. There is also some inconsistant data on the vasodialating qualities of estrogen (Sullivan, 1974 and Brown et al., 1974) as opposed to it increasing coronary artery smooth muscle tone (Jaffe, 1977). Further examination of the effects of high and low estrogen levels is needed (Lancet, 1977). This study may be able to resolve some of the questions raised from previous data.

Materials and Methods

Animals and Hormonal Treatment

One month old, CD Strain, female rats (Charles River Breeding Labs.) were maintained on Purina Rat Chow and tap water ad libitum. All animals were housed in the laboratory at 70 ± 2°F with a fluorescent lighting schedule of 12 L : 12 D.

Hormonal and Surgical Treatment

Estradiol benzoate (Sigma Chemical Co.) was dissolved in cottonseed oil (Eastman Kodak Co.) and administered daily as 0.1 ml intraperitoneal (i.p.) injections for a period of 16 weeks as in Table 1.

The estradiol replacement hormone levels in this research were selected as a result of the studies of McPherson et al. (1974) and Eldridge et al. (1974). These researchers, utilizing castrate animals, based their "normal" replacement levels on both bioassay replacement weights of accessory sex glands and radioimmunoassay suppression studies of pituitary gonadotrophins using daily exogenous injections of estradiol benzoate.

Demonstration of Coronary Blood Flow

A. The cannulation of the left femoral artery

The rats were anesthetized with 5 mg/100 gms of sodium phenobarbital

Table 1. Surgical and Hormonal Treatment of the Four Treatment Groups.

Group	Gender (n)	Surgical Treatment	Daily Hormonal Treatment
1	Female (10)	ovariectomized	Oil (0.1 ml)
2	Female (10)	None	None
3	Female (10)	ovariectomized	4.0 µg estradiol benzoate in oil (0.1 ml)
4	Female (10)	ovariectomized	40.0 µg estradiol benzoate in oil (0.1 ml)

administered intraperitoneally (i.p.) and the left femoral artery was then cannulated. A longitudinal cut of approximately two inches was made between the abdomen and the left leg to expose the femoral artery. A branch of the femoral artery, the deep femoral artery, was clamped and cut to make it easier to manipulate the femoral artery. Next, connective tissue was cleared away until the femoral artery became clearly visible. The artery was mobilized by loosening it from concommitant neural tissue. Forceps were used to elevate the artery to free it from the vein until about 0.6 cm of artery was free. Suture string and hemostats were used to loosely ligate the artery. The sutures were looped around the artery, pulled and then clamped with hemostats to supply the proper tension. This was done to both the proximal and distal ends of the artery in order to elevate the artery, making it clearly visible and easier to reach. In addition, the increase in local resistance slowed blood flow into this region of the artery. Iris scissors were used to make a small angular cut at the distal end of the elevated artery. At this point the artery was again checked for any excess connective tissue since its presence would hamper the insertion of the tube. When blood began to seep from the cut in the artery, the points of the scissors were inserted into the artery and reciprocated in and out several times in order to make the incision clear and visible. Next, while holding tight the distal suture with the wrist to prevent the artery from moving, the beveled end of PE 50 tubing (Clay Adams, Parsippany, N.J.), cut to approximately two and one half feet and filled with heparinized saline, was inserted into the artery. Pressure was still being placed on the proximal end to prevent loss of blood. After the tube was situated, it was inserted further and more securely into the artery by using a forcep on the tube in the artery and a forcep on the

tube outside the artery. Once in place, the tube was securely tied into the artery. A syringe withdrawal pump (Harvard apparatus, Millis, Mass.) connected to the tube was then started for a few seconds to withdraw a minimal amount of blood. The tube was then observed to ascertain the presence of a pulse. This insured a clear and unobstructed flow during the reference sample withdrawal period.

B. Microsphere injection

Styrene-divinyl benzene copolymer microspheres (15 ± 3 μm diameter, density approximately 1.38/cc, (New England Nuclear, Boston, Mass.) labeled with cobalt-57 were used. They are non-biodegradable and essentially no activity can be leached form the labeled spheres (0.15% leach in 5 ml saline after 15 days at room temperature; a sample giving 100,000 CPMs gives less than 2.5% leach in plasma after 48 hours at 38°C). The microspheres also will not aggregate. The size and density chosen was that which was as close to the diameter (7-8 μm) and density ($1.01/cm^3$) of normal red blood cells as possible. Thus the microspheres should circulate as normal blood cells through body tissue until they became trapped in the microvasculature. The microspheres were drawn into a 1 ml disposable tuberculin syringe (Rakûsan and Blahitka, 1974). After the bottle had been agitated by vortex for 30 seconds to suspend all the microspheres equally in the solution, a bolus of 0.05 ml was withdrawn from the stock solution. The bolus contained approximately 100,000 microspheres.

C. Cardiac Puncture

The microspheres were injected via cardiac puncture (Rakûsan and Blahitka, 1974) using a 2.5 cm, 23 gauge needle. "Penetration was made with the needle in an area on the left side of the closed thorax between the fifth and sixth ribs in an area slightly to the left and above the region where the xyphoid process joins the main body of the sternum" (Rakûsan and Blahitka, 1974). To make sure the needle was located properly inside the left ventricle, a small amount of blood was withdrawn and then the microspheres were quickly injected. The withdrawal pump was started immediately before the cardiac puncture and remained on for 120 seconds after the injection. A withdrawal rate of 0.0383 ml/min was used to withdraw blood from the femoral artery with a 2.0 ml silicone-treated glass syringe. After this reference sample was obtained, the animals were sacrificed and the heart removed.

D. Ventricle procurement

The atria were excised and discarded. The right ventricle was then separated from the left ventricle and septum by pinning the heart to a block of wax and carefully pulling and cutting the right ventricle from the septum (Martin et al., 1972). The left ventricle was then cut from the anterior end to the apex and opened (Figure 2). Both the right and left ventricles were dipped twice in 50 ml of saline solution and blotted twice on filter paper to remove any residual blood or microspheres not lodged in ventricular microvasculature. The ventricles were then weighed on a Mettler balance (Model PC 4400). The kidneys were also removed and weighed to demonstrate equal bilateral microsphere

distribution. The ventricles, kidneys and PE tubing containing the reference sample were placed in vials and counted by a Beckman Gamma Counter (Model 8000).

E. Blood flow calculations

Since the labeled microspheres were quickly injected into the left ventricle, they were rapidly mixed with the blood and were distributed proportionally to blood flow: N/c.o. = R, where c.o. is the cardiac output, N is the administered dose of activity, and R is a constant. Since the microspheres trapped in an organ had a count N (counts/minute) and the arterial flow to that organ is F (ml/minute), it follows that N/F = R. The withdrawal pump, which removes peripheral arterial blood at a known rate, represents an "artificial organ." If Np (counts/minute) are located in the blood collected by the withdrawal pump and if the pump has a withdrawal of Fp (ml/minute) then Np/Fp = N/F. After counting the activity ("N") in the portions of the heart, only ("F") remains unknown and can be easily calculated (Pharmacia, 1975 and Bartrum et al., 1974), see Figure 3.

Whole heart blood flow was calculated by taking into account the difference in mass of right and left ventricular myocardium and thus the difference in blood flow each contributed to the whole.

Figure 2. Right and left ventricular procurement.

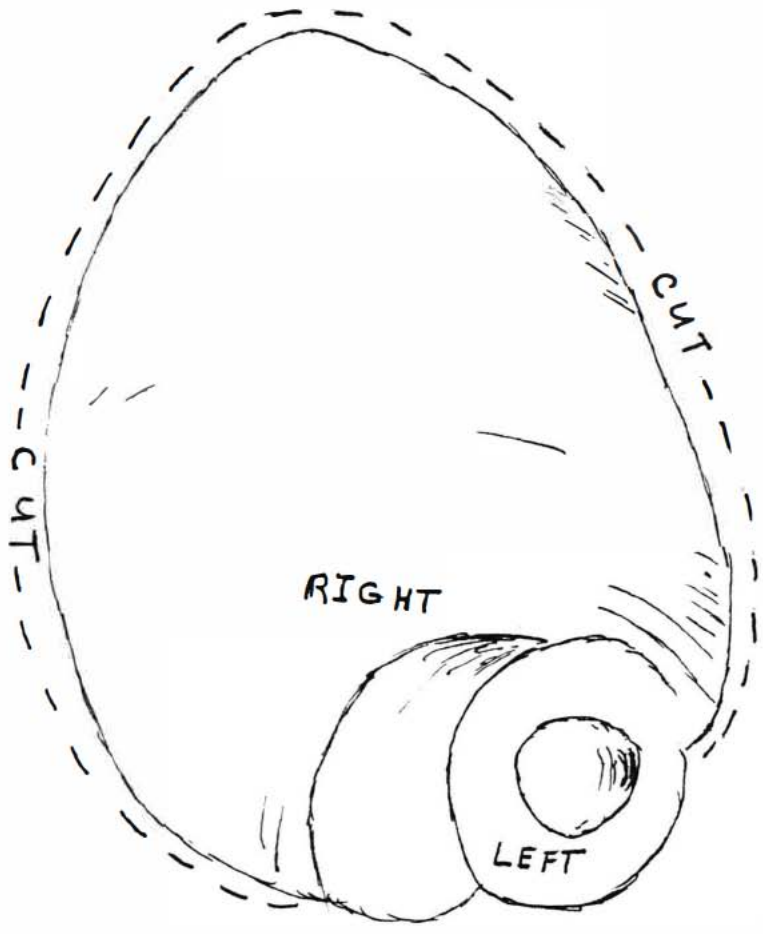

CUT
CUT
RIGHT
LEFT

Figure 3. Blood flow equation.

Fp = (ml/min) pump withdrawl rate

N = (counts/min) of micropheres trapped in organ

Np = (counts/min) of blood collected by withdrawal pump

F = (ml/min) of arterial blood flow to organ

$$\frac{N_p}{F_p} = \frac{N}{F}$$

$$F = \frac{F_p N}{N_p}$$

Results

Renal Blood Flow

The kidneys which were removed from the rats were used to ascertain complete mixing and equal dispersion of microspheres in the blood as it was ejected from the left ventricle. The blood flow from both right and left kidneys was calculated (ml/min/gm) from all four groups and compared (Table 1). The results demonstrated no significant difference in microsphere trapping by the two kidneys ($P > 0.05$). This served as confirmation of proportional bilateral distribution of microspheres in the systemic circulation.

Right and Left Ventricular Blood Flow

Ventricular blood flow was calculated as ml/min/gm of tissue. Figure 4 compares right and left ventricular blood flow for the four different treatment groups. All the right and left ventricular blood flows were compared to see if blood flow was increased in one ventricle over the other and no significance ($P > 0.05$) was found between them (Table 2). Differences in right and left ventricular blood flow also were found to be not significant ($P > 0.05$) as far as any interaction taking place between right or left and any one of the treatments (Table 2).

Whole Heart Blood Flow

Blood flow in whole heart was calculated as ml/min/gm for all four

Table 2. Analysis of variance comparing the mean blood flow of the right and left kidneys of the four groups. NS (P>.05).

Source	DF*	Sum of Squares	Mean Square	Type IV SS**	F-Value	PR > F
Side***	1	——	——	0.02	0.01	0.9117
Error	29	36.99	1.28	——	——	——

*DF = Degrees of Freedom

**SS = Sum of Squares

***Side = Right and Left Kidneys

Figure 4. Effect of estrogen on blood flow in right and left ventricles in the four treatment groups. (Each bar represents group mean ± SE).

G_1 = ovariectomized oil group

G_2 = normal group

G_3 = ovariectomized 4.0 µg estrogen group

G_4 = ovariectomized 40.0 µg estrogen group

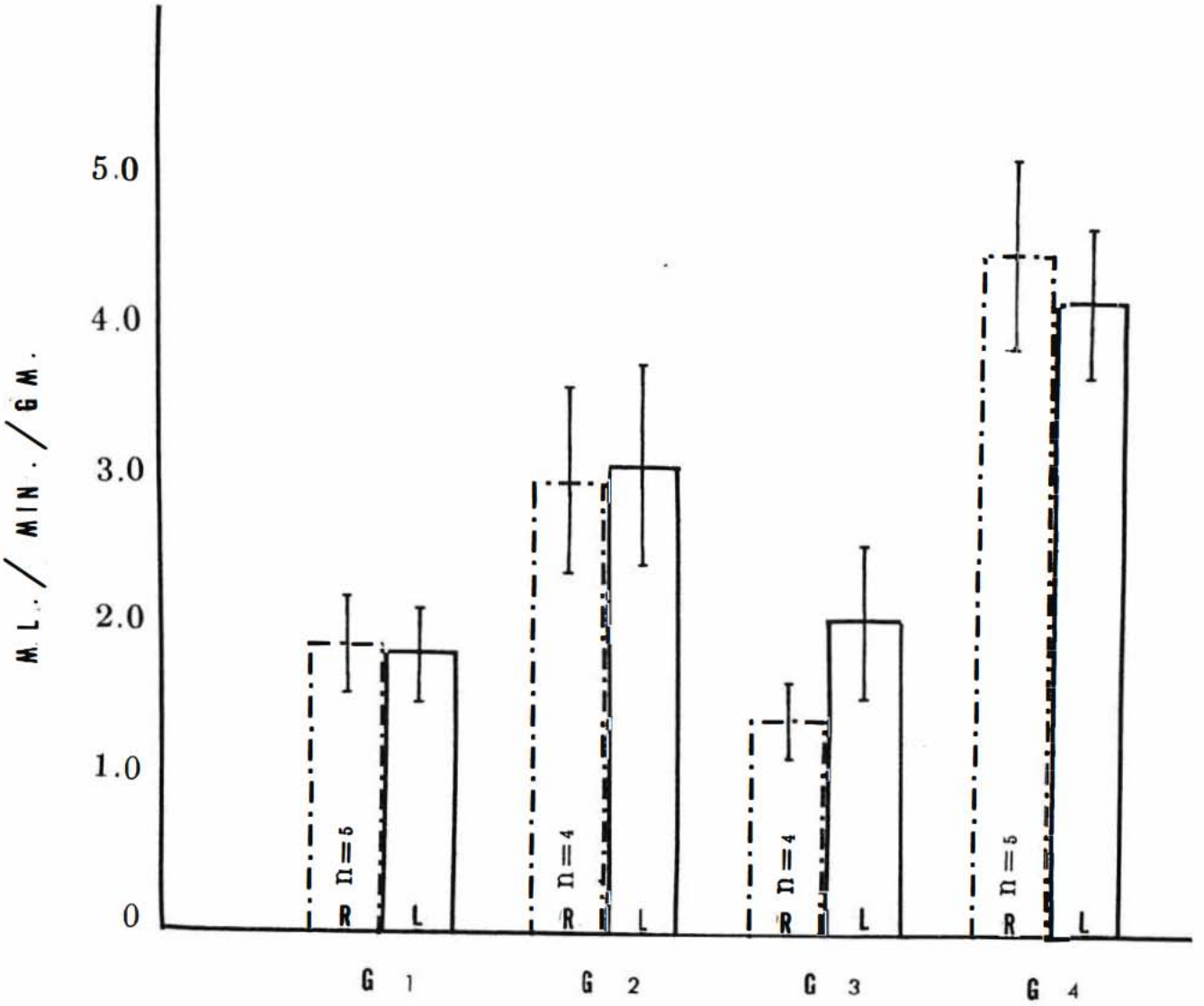

ML. / MIN. / GM.
5.0
4.0
3.0
2.0
1.0
0
n=5
R
L
n=4
R
L
n=4
R
L
n=5
R
L
G 1
G 2
G 3
G 4

Table 3. Analysis of variance comparing the mean blood flow of right and left ventricular myocardium (and if effected by the treatment) of the four groups. Both are NS(P>.05)

Source	DF*	Sum Of Squares	Mean Square	Type IV SS**	F-Value	PR > F
Side***	1	——	——	0.41	0.37	0.5493
Side and Treatment	3	——	——	1.22	0.36	0.7813
Error	28	31.4	1.12	——	——	——

*DF = Degrees of Freedom

**SS = Sum of Squares

***Side = Right and Left Ventricles

Figure 5. Effect of estrogen on blood flow in whole hearts (right plus left ventricle) in the four treatment groups. (Each bar represents group mean ± SE).

G_1 = ovariectomized oil group

G_2 = normal group

G_3 = ovariectomized 4.0 µg estrogen group

G_4 = ovariectomized 40.0 µg estrogen group

ML./MIN./GM.
5.0
4.0
3.0
2.0
1.0
0
n = 5
n = 4
n = 4
n = 5
G 1
G 2
G 3
G 4

groups and shown in Figure 5. It is obvious that the greatest mean blood flow is seen in G_4 (the 40.0 µg group), followed by G_2 (normals), G_3 (4.0 µg) and G_1 (oil). Table 3 shows by analysis of variance that the mean blood flow in whole heart in the four groups is significantly different ($P < 0.01$).

Results of whole heart blood flow are shown in Table 4. The mean blood blow of G_4 was 131% greater than that of G_1 ($P < 0.01$). However, the blood flow rates of G_1 and G_3 are not significantly different. The mean flow rate of G_4 is greater than that of G_3 ($P < 0.01$). Estrogen appears to increase blood flow in G_2 by 65% over G_1 when compared but the difference is not significant ($P > 0.05$). The blood flow means of G_2 are higher than that of G_3 when compared but this was not a significant difference. G_4 also has a larger mean blood flow than G_2 but once more the difference is not significant.

In summary, G_4 had the greater blood flow rates among the four groups in the study. It also can be infered from these data that the blood flow rates among the groups are proportionally increased as the dosage of estrogen administered is also increased ($r_{xy} = 0.82$).

Table 4. Analysis of variance comparing the mean blood flow of whole hearts of the four groups. Significant (P<.01).

Source	DF*	Sum of Squares	Mean Square	Type IV SS**	F-Value	PR > F
Treatment	3	——	——	18.99	6.21	0.006
Error	14	14.26	1.02	——	——	——

*DF Degrees of Freedom

**SS = Sum of Squares

Table 5. Group Comparisons and Significance Levels of Whole Heart Myocardial Blood Flow.

Groups Compared	Difference and Significance	Whole Heart Blood Flow (ml/min/gm)	% Increase In Blood Flow
Group 1 - Group 2	difference	1.22	65%
	significance	NS*	NS*
Group 1 - Group 3	difference	.108	5.7%
	significance	NS*	NS*
Group 1 - Group 4	difference	2.46	131%
	significance	<.01	<.01
Group 2 - Group 3	difference	1.11	56%
	significance	NS*	NS*
Group 2 - Group 4	difference	1.24	40%
	significance	NS*	NS*
Group 3 - Group 4	difference	2.35	111%
	significance	<.01	<.01

*NS indicates that P>.05 as analyzed by Duncan's multiple range test

Discussion

This study was devised to determine whether or not estrogen effects blood flow in ventricular myocardial tissue by either decreasing or increasing it. Rather than the more complicated method of quantitating this by injection of microspheres via retrograde cannulation (McDevitt and Nies, 1976) which may result in aortic ejection blockage and possible aortic damage, the method of Rakûsan and Blahitka (1974) was used, which employs injection of microspheres directly into the left ventricle via cardiac puncture. The data of the experiment indicate that estrogen does effect and can increase blood flow to the ventricular myocardium of the rat, especially at the 40.0 µg dosage. This is supported by the significant differences found in the blood flow (ml/min/gm) means between the 40.0 µg estrogen-injected castrates and the oil-injected castrates and also the 4.0 µg estrogen-injected castrates and the 40.0 µg estrogen-injected castrates in the study.

As far as estrogen effecting blood flow differently in the right or left ventricle, no significant difference was found either related or unrelated to the treatment of the groups.

The discrepancy in mean blood flow between the 4.0 µg group and the normal rats may have resulted from the level chosen as the normal physiological replacement dosage of estrogen. One would expect that the normal physiological replacement group, the 4.0 µg group, would position its blood flow closer to the mean of the normal group. This

dosage was determined to be the normal replacement amount by McPherson et al. (1974) and Eldridge et al. (1974) for younger and smaller rats (250 gm). In the present study at the time of sacrifice, however, the rats were older and weighed between 250 - 350 gm. Thus, it would take a greater amount of hormone since the hormone would be diluted by a greater mass of tissue. So in older rats the replacement dosage lies, most likely, slightly above 4.0 µg of estrogen.

The 40.0 µg group results contradict earlier statements made by Jaffe (1977) that estrogen treatment diminishes coronary blood flow and increases coronary smooth muscle tone. This statement was made before any attempt had been made to study the effects of estrogen on the coronary arteries directly. The present study supports Sullivan (1974), Brown et al. (1974) and Silva De Sa' and Meirelles (1977) with their conclusion that estrogen is a vasodialator in various vascular beds.

These findings may be the mechanism by which estrogen provides protection to premenopausal women against myocardial infarction (Higano et al., 1963 and Stamler, 1967). This also may account for the protection given to men following estrogen therapy after a myocardial infarction (Stamler et al., 1963).

The present study indicates that estrogen is acting on the vessels of the heart, possibly via estrogen target sites in coronary vasculature (Stumpf et al., 1977) thus increasing the capacity of the coronary microvasculature to perfuse the heart muscle.

This study reveals one possible mechanism by which estrogen acts on the coronary arteries of the rat heart. Estrogen's past

history tends to be one of uncertainty and contradictions concerning its pharmacological effects on the circulatory system. Estrogen has been attributed to producing such side effects as heart disease and stroke to name a few. A closer look at different levels of estrogen and its effect on the circulatory system should be undertaken to evaluate if estrogen can be of better use clinically. Also it should be evaluated to see possibly if some of estrogen's so-called side effects are induced directly by its effects on blood vessel walls or by manifesting its harmful effects by some other mode of action. For example, increased platelet adhesion has been demonstrated in women taking pharmacological doses of estrogen in birth control medication. This study supports the judgement that estrogen probably provides protection from myocardial infarction and alters circulatory problems in the heart by way of increasing blood flow if administered within physiological ranges.

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Vita